invitrogen™
by life technologies™

pEF5/FRT/V5 Directional TOPO® Expression Kit

Five-minute, directional TOPO® Cloning of blunt-end PCR products into an expression vector containing the human EF-1α promoter and a C-terminal V5 epitope for use with the Flp-In™ System

Catalog no. K6035-01

Rev. Date: 7 July 2010
Manual part no. 25-0476

MAN0000265

User Manual

Table of Contents

Important Information

Shipping/Storage

The pEF5/FRT/V5 Directional TOPO® Expression Kit is shipped on dry ice. Each kit contains two boxes. Upon receipt, store as detailed below.

Box	Item	Storage
1	pEF5/FRT/V5-D-TOPO® Reagents	-20°C
2	One Shot® TOP10 chemically competent *E. coli*	-80°C

pEF5/FRT/V5-D-TOPO® Reagents

pEF5/FRT/V5-D-TOPO® reagents (Box 1) are listed below. **Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.**

Store Box 1 at -20°C.

Item	Concentration	Amount
pEF5/FRT/V5-D-TOPO® vector, TOPO®-adapted	15-20 ng/µl linearized plasmid DNA in: 45% glycerol 50 mM Tris-HCl, pH 7.5 (at 25°C) 0.25 mM EDTA 0.28 M NaCl 1 mM DTT 0.05% Triton X-100 50 µg/ml BSA 30 µM bromophenol blue	20 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Sterile Water	---	1 ml
T7 Promoter Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
BGH Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Expression Control Plasmid (pEF5/FRT/V5/GW-CAT)	0.5 µg/µl in TE Buffer, pH8	10 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8	10 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8	10 µl

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Important Information, continued

Sequences of the Primers

The table below provides the sequences of T7 Promoter and BGH Reverse sequencing primers. Two micrograms of each primer are supplied.

Primer	Sequence	pMoles Supplied
T7 Promoter	5´-TAATACGACTCACTATAGGG-3´	328
BGH Reverse	5´-TAGAAGGCACAGTCGAGG-3´	358

One Shot® TOP10 Reagents

The table below lists the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is 1 x 10^9 cfu/µg DNA.

Store Box 2 at -80°C.

Item	Composition	Amount
S.O.C. Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of TOP10

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Accessory Products

Introduction

The products listed in this section may be used with the pEF5/FRT/V5 Directional TOPO® Expression Kit. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 32).

Additional Products

Some of the products included in the pEF5/FRT/V5 Directional TOPO® Expression Kit as well as other reagents that may be used with the Flp-In™ System are available separately from Invitrogen. Ordering information is provided below.

Product	Amount	Catalog no.
Hygromycin	1 g	R220-05
Zeocin™	1 g	R250-01
	5 g	R250-05
pFRT/*lac*Zeo	20 µg, lyophilized in TE	V6015-20
pFRT/*lac*Zeo2	20 µg, lyophilized in TE	V6022-20
pOG44	20 µg, lyophilized in TE	V6005-20
Phosphate Buffered Saline, pH 7.4	500 ml	10010-023
One Shot® TOP10 Chemically Competent Cells	10 reactions	C4040-10
	20 reactions	C4040-03
	40 reactions	C4040-06
One Shot® TOP10 Electrocompetent Cells	10 reactions	C4040-50
	20 reactions	C4040-52
CAT Antiserum*	50 µl	R902-25

*The amount supplied is sufficient to perform 25 Western using 10 ml working solution per reaction.

Flp-In™ Expression Vectors

Additional Flp-In™ expression vectors are available from Invitrogen. For more information about each vector, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 32).

Product	Amount	Catalog no.
pcDNA5/FRT	20 µg, lyophilized in TE	V6010-20
pEF5/FRT/V5-DEST Gateway® Vector	6 µg	V6020-20
pcDNA5/FRT/V5-His TOPO® TA Expression Kit	1 kit	K6020-01
pSecTag/FRT/V5-His TOPO® TA Expression Kit	1 kit	K6025-01

continued on next page

Accessory Products, continued

Flp-In™ Host Cell Lines

For your convenience, Invitrogen has available several mammalian Flp-In™ host cell lines that stably express the *lacZ-Zeocin*™ fusion gene from pFRT/*lac*Zeo or pFRT/*lac*Zeo2. Each cell line contains a single integrated FRT site as confirmed by Southern blot analysis. The cell lines should be maintained in medium containing Zeocin™. For more information about each cell line, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 32).

Cell Line	Amount	Catalog no.
Flp-In™-293	3 x 10^6 cells, frozen	R750-07
Flp-In™-CV-1	3 x 10^6 cells, frozen	R752-07
Flp-In™-CHO	3 x 10^6 cells, frozen	R758-07
Flp-In™-BHK	3 x 10^6 cells, frozen	R760-07
Flp-In™-3T3	3 x 10^6 cells, frozen	R761-07
Flp-In™-Jurkat	3 x 10^6 cells, frozen	R762-07

Detection of Recombinant Proteins

Expression of your recombinant fusion protein can be detected using Anti-V5 antibodies available from Invitrogen. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods. Fluorescein isothiocyanate (FITC)-conjugated antibodies allow one-step detection in immunofluorescence experiments.

The amount of antibody supplied is sufficient for 25 Western blots or 25 immuno-staining reactions (FITC-conjugated antibody only).

Product	Epitope	Catalog no.
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991) GKPIPNPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-V5-FITC Antibody		R963-25

Introduction

Overview

Introduction

The pEF5/FRT/V5 Directional TOPO® Expression Kit combines the Flp-In™ System with TOPO® Cloning technology to provide a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector for targeted expression of the gene of interest in mammalian cell lines. Blunt-end PCR products clone directionally at greater than 90% efficiency, with no ligase, post-PCR procedures, or restriction enzymes required.

pEF5/FRT/V5 Directional TOPO® Vector

pEF5/FRT/V5-D-TOPO® is a 5.8 kb expression vector designed to facilitate rapid, directional TOPO® cloning and expression of PCR products using the Flp-In™ System available from Invitrogen. When cotransfected with the pOG44 Flp recombinase expression plasmid into a Flp-In™ mammalian host cell line, the pEF5/FRT/V5-D-TOPO® vector containing the PCR product of interest is integrated in a Flp recombinase-dependent manner into the genome. Features of the vector include:

- The human EF-1α promoter for high-level expression of your PCR product in a wide range of mammalian cells (Goldman *et al.*, 1996; Mizushima and Nagata, 1990) (see page 27 for a diagram)
- Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see page 9 for more information)
- C-terminal peptide containing the V5 epitope tag for detection of your recombinant protein
- FLP Recombination Target (FRT) site for Flp recombinase-mediated integration of the vector into the Flp-In™ host cell line (see next page for more information)
- Hygromycin resistance gene for selection of stable cell lines (Gritz and Davies, 1983) (see important note on page 3)

The control plasmid, pEF5/FRT/V5/GW-CAT, is included for use as a positive control for transfection and expression in the Flp-In™ host cell line of choice.

For more information about the Flp-In™ System, the pOG44 plasmid, and generation of the Flp-In™ host cell line, refer to the Flp-In™ System manual. The Flp-In™ System manual is supplied with the Flp-In™ Complete System (Catalog no. K6010-01) or the Flp-In™ Core System (Catalog no. K6010-02) and is also available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 32).

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Overview, continued

Flp Recombinase-Mediated DNA Recombination

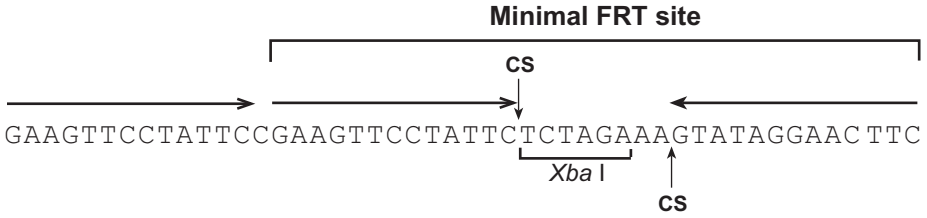
In the Flp-In™ System, integration of your pEF5/FRT/V5-D-TOPO® expression construct into the genome occurs via Flp recombinase-mediated intermolecular DNA recombination. The hallmarks of Flp-mediated recombination are listed below.

- Recombination occurs between specific FRT sites (see below) on the interacting DNA molecules
- Recombination is conservative and requires no DNA synthesis; the FRT sites are preserved following recombination and there is minimal opportunity for introduction of mutations at the recombination site
- Strand exchange requires only the small 34 bp minimal FRT site (see below)

For more information about the Flp recombinase and conservative site-specific recombination, refer to published reviews (Craig, 1988; Sauer, 1994).

FRT Site

The FRT site, originally isolated from *Saccharomyces cerevisiae*, serves as a binding site for Flp recombinase and has been well-characterized (Gronostajski and Sadowski, 1985; Jayaram, 1985; Sauer, 1994; Senecoff *et al.*, 1985). The minimal FRT site consists of a 34 bp sequence containing two 13 bp imperfect inverted repeats separated by an 8 bp spacer that includes an *Xba* I restriction site (see figure below). An additional 13 bp repeat is found in most FRT sites, but is not required for cleavage (Andrews *et al.*, 1985). While Flp recombinase binds to all three of the 13 bp repeats, strand cleavage actually occurs at the boundaries of the 8 bp spacer region (see figure below) (Andrews *et al.*, 1985; Senecoff *et al.*, 1985).



CS = cleavage site

FRT Site in pEF5/FRT/V5-D-TOPO®

The pEF5/FRT/V5-D-TOPO® vector contains a single FRT site immediately upstream of the hygromycin resistance gene for Flp recombinase-mediated integration and selection of the pEF5/FRT/V5-D-TOPO® construct following cotransfection of the vector (with pOG44) into a Flp-In™ mammalian host cell line. The FRT site serves as both the recognition and cleavage site for the Flp recombinase and allows recombination to occur immediately adjacent to the hygromycin resistance gene. The Flp recombinase is expressed from the pOG44 plasmid. For more information about pOG44, refer to the pOG44 manual or the Flp-In™ System manual.

continued on next page

Overview, continued

Important

The hygromycin resistance gene in pEF5/FRT/V5-D-TOPO® lacks a promoter and an ATG initiation codon; therefore, transfection of the pEF5/FRT/V5-D-TOPO® plasmid alone into mammalian host cells will **not** confer hygromycin resistance to the cells. The SV40 promoter and ATG initiation codon required for expression of the hygromycin resistance gene are integrated into the genome (in the Flp-In™ host cell line) and are only brought into the correct proximity and frame with the hygromycin resistance gene through Flp recombinase-mediated integration of pEF5/FRT/V5-D-TOPO® at the FRT site. For more information about the generation of the Flp-In™ host cell line and details of the Flp-In™ System, refer to the Flp-In™ System manual.

How Directional TOPO® Cloning Works

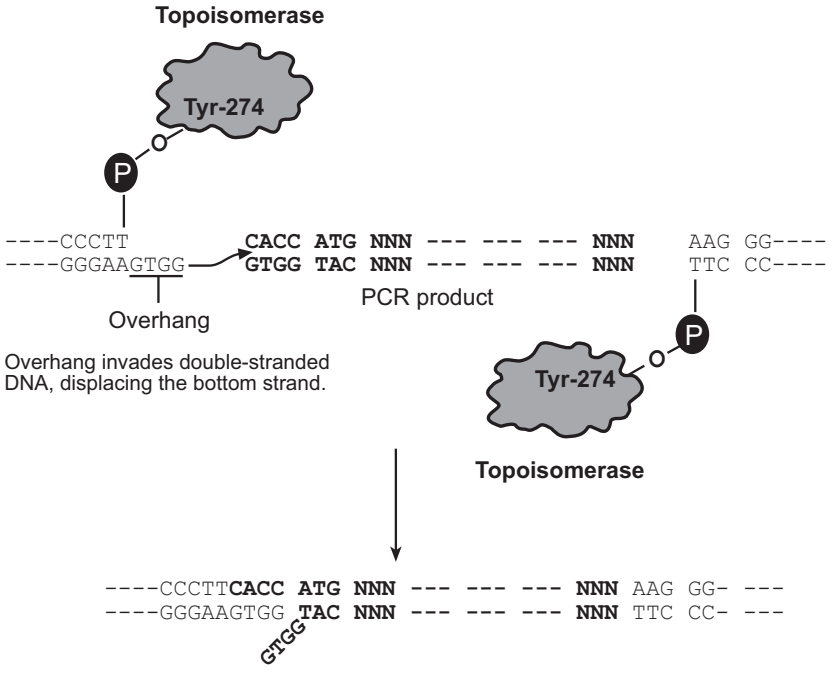
How Topoisomerase I Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3′ single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000; Southern *et al.*, 1991). This single-stranded overhang is identical to the 5′ end of the TOPO®-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



Experimental Outline

Experimental Outline

The table below describes the general steps needed to clone and express your gene of interest. For more details, refer to the pages indicated.

Step	Action	Page
1	Design PCR primers to clone your gene of interest in frame with the C-terminal V5 epitope tag (if desired). Consult the diagram on page 8 to help you design your PCR primers.	6-8
2	Produce your blunt-end PCR product.	9
3	TOPO® Clone your PCR product into pEF5/FRT/V5-D-TOPO® and transform into One Shot® TOP10 *E. coli*. Select transformants on LB plates containing 50-100 µg/ml ampicillin.	10-14
4	Analyze transformants by restriction digestion or PCR.	15
5	Select a transformant with the correct restriction pattern and sequence it to confirm that your gene is cloned in frame with the C-terminal V5 epitope tag (if desired).	15
6	Cotransfect your pEF5/FRT/V5-D-TOPO® construct and pOG44 into the Flp-In™ host cell line using your method of choice and select for hygromycin resistant clones (see the Flp-In™ System manual for more information).	17-18
7	Assay for expression of your protein of interest.	19-20

Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Consider the following when designing your PCR primers.

- Sequences required to facilitate directional cloning
- Sequences required for proper translation initiation of your PCR product
- Whether or not you wish your PCR product to be fused in frame with the C-terminal V5 epitope tag

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the following points below. Refer to page 8 for a diagram of the TOPO® Cloning site for pEF5/FRT/V5-D-TOPO®.

- To enable directional cloning, the forward PCR primer **MUST** contain the sequence, CACC, at the 5′ end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in pEF5/FRT/V5-D-TOPO®.
- Make sure your sequence of interest includes a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990; Southern *et al.*, 1991). An example of a Kozak consensus sequence is **(G/A)**NNATG**G**. Other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is underlined.

  Note: If your sequence of interest does not contain an initiation codon within the context of a Kozak sequence, design the forward PCR primer to contain a Kozak sequence at the 5′ end of the primer (see **Example** below).

Example of Forward Primer Design

Below is the sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer. The ATG initiation codon is underlined.

DNA sequence: 5′-ATG GGA TCT GAT AAA

Proposed Forward PCR primer: 5′-C ACC ATG GGA TCT GAT AAA

If you design the forward PCR primer as noted above, then the ATG initiation codon falls within the context of a Kozak sequence (see boxed sequence), allowing proper translation initiation of the PCR product.

Note

The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a functional codon.

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Designing PCR Primers, continued

Guidelines to Design the Reverse Primer

When designing your reverse PCR primer, consider the points below. Refer to page 8 for a diagram of the TOPO® Cloning site for pEF5/FRT/V5-D-TOPO®.

- **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 50%, increasing the likelihood of your ORF cloning in the opposite orientation (see Example #1 below).** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch.
- If you wish to fuse your PCR product in frame with the C-terminal V5 epitope tag, design the reverse PCR primer to remove the native stop codon in the gene of interest (see **Example #2** on the next page).
- If you do NOT wish to fuse your PCR product in frame with the C-terminal V5 epitope tag, include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site (see **Example #2** on the next page).

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag. The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′

One solution is to design the reverse PCR primer to start with the codon just upstream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′
Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5′

Another solution is to design the reverse primer so that it hybridizes just downstream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine.

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Designing PCR Primers, continued

Example #2 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. The stop codon is underlined.

…GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG-3′

- To fuse the ORF in frame with a C-terminal tag, remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

  5′-TGC AGT CGT CGA GTG CTC CGA CTT-3′

  This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with a C-terminal tag.
- If you don't want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

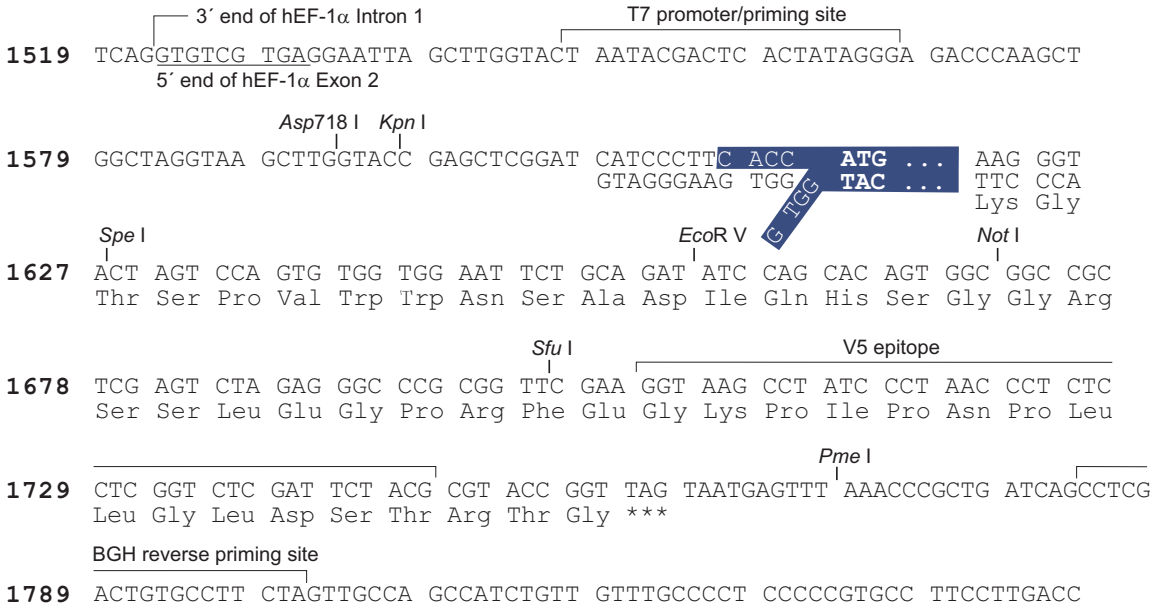
  5′-CTA TGC AGT CGT CGA GTG CTC CGA CTT-3′

Important

- Remember that pEF5/FRT/V5-D-TOPO® accepts blunt-end PCR products.
- Do not add 5′ phosphates to your primers for PCR. This will prevent ligation into pEF5/FRT/V5-D-TOPO®.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

TOPO® Cloning Site for pEF5/FRT/V5-D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pEF5/FRT/V5-D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. **The sequence of pEF5/FRT/V5-D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 32).**



Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, produce your blunt-end PCR product using any thermostable, proofreading polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Materials Supplied by the User

You will need the following reagents and equipment for PCR.

Note: dNTPs (adjusted to pH 8) are provided in the kit.

- Thermocycler and thermostable, proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- DNA template and primers for PCR product

Producing Blunt-End PCR Products

Set up a 25 µl or 50 µl PCR reaction using the guidelines below.

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- After cycling, place the tube on ice or store at -20ºC for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations to optimize your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see pages 25-26).
- Estimate the concentration of your PCR product. You will use this information when setting up your TOPO® Cloning reaction (see **Amount of PCR Product to Use in the TOPO® Cloning Reaction**, next page for details).

Performing the TOPO® Cloning Reaction

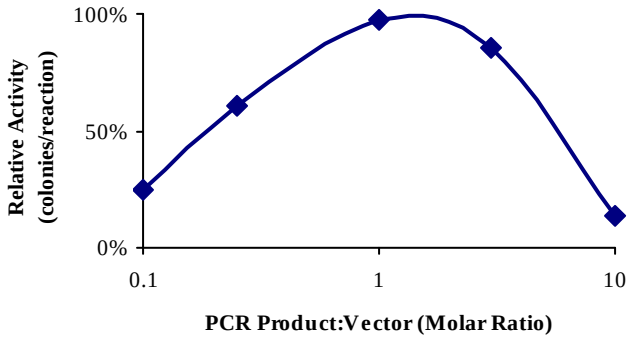
Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pEF5/FRT/V5-D-TOPO® vector and transform the recombinant vector into TOP10 *E. coli*. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transforming One Shot® TOP10 Competent Cells** (pages 12-13) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 23-24 in parallel with your samples.

Amount of PCR Product to Use in the TOPO® Cloning Reaction

When performing directional TOPO® Cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. **To obtain the highest TOPO® Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector (see figure below).** Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1 (see figure below). These results are generally obtained if too little PCR product is used (*i.e.* PCR product is too dilute) or if too much PCR product is used in the TOPO® Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Tip: For the pEF5/FRT/V5-D-TOPO® vector, using 1-5 ng of a 1 kb PCR product or 5-10 ng of a 2 kb PCR product in a TOPO® Cloning reaction generally results in a suitable number of colonies.



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Performing the TOPO® Cloning Reaction, continued

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page vii for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed below.

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.* **Reminder:** For optimal results, be sure to use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.

Note: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 µl

*Store all reagents at -20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® TOP10 Competent Cells**, next page.

   Note: You may store the TOPO® Cloning reaction at -20°C overnight.

Transforming One Shot® TOP10 Competent Cells

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pEF5/FRT/V5-D-TOPO® construct into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation, however, you may also transform electrocompetent cells (see page vii for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Materials Supplied by the User

In addition to general microbiological supplies (*e.g.* plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 50-100 µg/ml ampicillin (two for each transformation)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned in the correct orientation. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparing for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm LB plates containing 50-100 µg/ml ampicillin at 37°C for 30 minutes.
- Thaw **on ice** 1 vial of One Shot® TOP10 cells from Box 2 for each transformation.

continued on next page

Transforming One Shot® TOP10 Competent Cells, continued

One Shot® TOP10 Chemical Transformation Protocol

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 11 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 15).

Transformation by Electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 11 into a 0.1 cm cuvette containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.**
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see the next page.
3. Immediately add 250 µl of room temperature S.O.C. medium.
4. Transfer the solution to a 15 ml snap-cap tube (*e.g.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the ampicillin resistance gene.
5. Spread 20-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 15).

continued on next page

Transforming One Shot® TOP10 Competent Cells, continued

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB or SOB medium containing 50-100 µg/ml ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing

You may sequence your construct to confirm that your gene is cloned in the correct orientation and in frame with the C-terminal V5 epitope tag, if desired. The T7 Promoter and BGH Reverse primers are included in the kit to help you sequence your insert. Refer to the diagram on page 8 for the location of the primer binding sites.

Important

If you download the sequence for pEF5/FRT/V5-D-TOPO® from our Web site, note that the overhang sequence (GTGG) will be shown already hybridized to CACC. No DNA sequence analysis program allows us to show the overhang without the complementary sequence.

Analyzing Transformants by PCR

You may analyze positive transformants using PCR. For PCR primers, use a combination of the T7 Promoter primer or the BGH Reverse primer and a primer that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed

PCR SuperMix High Fidelity (Invitrogen, Catalog no. 10790-020)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5 colonies and resuspend them individually in 50 µl of the PCR cocktail from Step 1, above.
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

continued on next page

Analyzing Transformants, continued

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 23-24. These reactions will help you trouble-shoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colony on LB plates containing 50-100 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50-100 µg/ml ampicillin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Generating the Flp-In™ Expression Cell Line

Introduction

This section provides general information for cotransfecting your pEF5/FRT/V5-D-TOPO® construct and pOG44 plasmid into your mammalian Flp-In™ host cell line to generate your stable Flp-In™ expression cell line. We recommend that you include the pEF5/FRT/V5/GW-CAT positive control vector and a mock transfection (negative control) in your experiment to evaluate your results. Specific guidelines and protocols as well as detailed information about pOG44 and generation of the Flp-In™ host cell line can be found in the Flp-In™ System manual.

Several Flp-In™ host cell lines which stably express the *lacZ-Zeocin*™ fusion gene and contain a single integrated FRT site are available from Invitrogen (see page viii for ordering information). For more information on these cell lines, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 32).

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (*e.g.* HeLa, CHO), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). We typically use lipid-based transfection reagents to introduce Flp-In™ expression constructs into Flp-In™ host cell lines. If you wish to use a lipid-based reagent for transfection, we recommend using Lipofectamine™ 2000 Reagent available from Invitrogen (Catalog no. 11668-027). For more information about Lipofectamine™ 2000 Reagent and other transfection reagents available from Invitrogen, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 32).

continued on next page

Generating the Flp-In™ Expression Cell Line, continued

Positive Control

pEF5/FRT/V5/GW-CAT is provided as a positive control vector for mammalian cell transfection and expression (see page 30 for a map) and may be used to assay for recombinant protein expression levels in your Flp-In™ host cell line. Cotransfection of the positive control vector and pOG44 into your Flp-In™ host cell line allows you to generate a stable cell line expressing chloramphenicol acetyl transferase (CAT) at the same genomic locus as your gene of interest. If you have several different Flp-In™ host cell lines, you may use the pEF5/FRT/V5/GW-CAT control vector to compare protein expression levels between the various cell lines.

To propagate and maintain the plasmid:

1. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α, or equivalent.
2. Select transformants on LB agar plates containing 50-100 μg/ml ampicillin.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

Hygromycin B

The pEF5/FRT/V5-D-TOPO® vector contains the hygromycin resistance gene (Gritz and Davies, 1983) for selection of transfectants with the antibiotic, hygromycin B (Palmer *et al.*, 1987). When added to cultured mammalian cells, hygromycin B acts as an aminocyclitol to inhibit protein synthesis. Hygromycin B is available separately from Invitrogen (Catalog no. R220-05). For instructions to handle and store hygromycin B, refer to the Flp-In™ System manual.

Determination of Hygromycin Sensitivity

Before generating a stable Flp-In™ expression cell line, we recommend that you generate a kill curve to determine the minimum concentration of hygromycin required to kill your untransfected Flp-In™ host cell line. Generally, concentrations between 10 and 400 μg/ml hygromycin are required for selection of most mammalian cell lines. General guidelines for performing a kill curve are provided in the Flp-In™ System manual.

Important

The hygromycin resistance gene in pEF5/FRT/V5-D-TOPO® lacks a promoter and an ATG initiation codon; therefore, transfection of the pEF5/FRT/V5-D-TOPO® plasmid alone into mammalian host cells will **not** confer hygromycin resistance to the cells. The SV40 promoter and ATG initiation codon required for expression of the hygromycin resistance gene are integrated into the genome (in the Flp-In™ host cell line) and can only be brought into the correct proximity and frame with the hygromycin resistance gene through Flp recombinase-mediated integration of pEF5/FRT/V5-D-TOPO® at the FRT site.

Assaying for Expression

Introduction

You may use a functional assay to detect the protein encoded by your PCR product or a Western blot analysis if you have an antibody to the protein. If you have elected to express your PCR product as a fusion to the V5 epitope, you may use antibodies to the V5 epitope to detect the fusion protein (see below).

Note

Your gene of interest will be expressed from pEF5/FRT/V5-D-TOPO® under the control of the human EF-1α promoter. Once you have generated the Flp-In™ expression cell line, your recombinant fusion protein will be constitutively expressed.

Detection of Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein by Western blot analysis, you may use the Anti-V5 antibodies available from Invitrogen (see page viii for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a V5 epitope tag. The ready-to-use WesternBreeze™ Chromogenic Kits and WesternBreeze™ Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 32).

Preparation of Cell Lysates

To detect your fusion protein by Western blot, you will need to prepare a cell lysate from transfected cells. A sample protocol is provided below. Other protocols are suitable. To lyse cells:

1. Wash cell monolayers (~5 x 10^5 to 1 x 10^6 cells) once with phosphate-buffered saline (PBS, see page vii for ordering information).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend in 50 µl Cell Lysis Buffer (see the **Appendix**, page 31 for a recipe). Other cell lysis buffers are suitable. Vortex.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells.

   Note: You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
5. Centrifuge the cell lysate at 10,000 x g for 10 minutes at +4°C to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration.

   Note: Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Add SDS-PAGE sample buffer (see the **Appendix**, page 31 for a recipe) to a final concentration of 1X and boil the sample for 5 minutes.
7. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese (see the next page). Use the appropriate percentage of acrylamide to resolve your fusion protein.

continued on next page

Assaying for Expression, continued

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. The NuPAGE® Gel System avoids the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 32).

Note

The C-terminal peptide tag containing the V5 epitope will add approximately 5 kDa to the size of your protein.

Assay for CAT Protein

If you use pEF5/FRT/V5/GW-CAT as a positive control vector, you may assay for CAT expression using your method of choice. Note that CAT is fused to the C-terminal V5 epitope tag, so you can use Western blot analysis and an Anti-V5 antibody to detect expression of CAT. CAT Antiserum is also available separately from Invitrogen (see page vii for ordering information). Other commercial kits are available for assaying CAT expression. The molecular weight of the CAT/V5 fusion protein is approximately 30 kDa.

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions in parallel with your samples (see pages 23-24).

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	• Dilute the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR product too dilute	• Concentrate the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence CACC at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
	Used *Taq* polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Long PCR product	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (*i.e.* does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of choice. • Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Use a0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.

continued on next page

Troubleshooting, continued

TOPO® Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Large percentage of inserts cloned in the incorrect orientation	Incorrect PCR primer design	Make sure that the forward PCR primer contains the sequence CACC at the 5′ end.
	Reverse PCR primer is complementary to the GTGG overhang at the 5′ end	Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
Large number of incorrect inserts cloned	PCR reaction contains artifacts (*i.e.* does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of choice. • Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence CACC at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at -80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	One Shot® transformation protocol not followed correctly	Follow the One Shot® transformation protocol provided on page 13.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using this product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 50-100 µg/ml ampicillin.

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile Water	41.5 µl
Thermostable polymerase (1-2.5 units/µl)	1 µl
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible.
5. Estimate the concentration of the PCR product, and adjust as necessary such that the amount of PCR product used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (*i.e.* 0.5:1 to 2:1). Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

Performing the Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pEF5/FRT/V5-D-TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below. If you plan to transform *E. coli* using electroporation, **DO NOT** include the salt solution.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pEF5/FRT/V5-D-TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® TOP10 cells (page 13).
4. Spread 100-200 µl of each transformation mix onto LB plates containing 50-100 µg/ml ampicillin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with *Not* I. The table below lists the digestion patterns that you should see for inserts that are cloned in the correct orientation or reverse orientation.

Orientation	Expected Fragments (bp)
Correct orientation	682, 5868
Reverse orientation	156, 6394
Empty vector	5831

Greater than 90% of the colonies should contain the 750 bp insert in the correct orientation. Relatively few colonies should be produced in the vector-only reaction.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® TOP10 competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 13. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA.

Gel Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you wish to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Three simple protocols are provided below.

Note

Note that cloning efficiency may decrease with purification of the PCR product (*e.g.* PCR product too dilute). You may wish to optimize your PCR to produce a single band (see **Producing Blunt-End PCR Products**, page 9).

Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.

   Note: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.
3. Add 1.5 volumes Binding Buffer.
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on page 11.

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 11) Be sure to make the gel slice as small as possible for best results.

continued on next page

Gel Purifying PCR Products, continued

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 11.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into One Shot® TOP10 cells using the method on page 13.

Note

Note that the cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

Human EF-1α Promoter

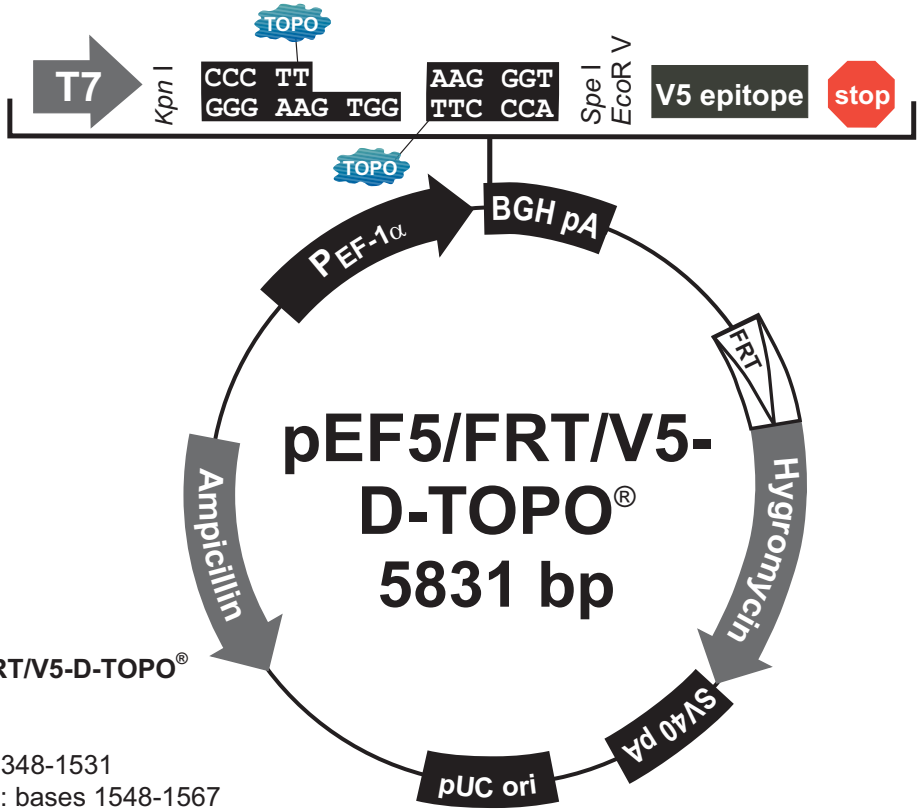
Description

The diagram below shows the features of the human EF-1α promoter (Mizushima and Nagata, 1990) used in the pEF5/FRT/V5-D-TOPO® vector. Features are marked as described in Uetsuki *et al*., 1989.

Map and Features of pEF5/FRT/V5-D-TOPO®

pEF5/FRT/V5-D-TOPO® Map

The figure below shows the features of pEF5/FRT/V5-D-TOPO® vector. **The complete sequence of pEF5/FRT/V5-D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 32).**



Comments for pEF5/FRT/V5-D-TOPO®
5831 nucleotides

EF-1α promoter: bases 348-1531
T7 promoter/priming site: bases 1548-1567
TOPO® recognition site 1: bases 1612-1616
Overhang: bases 1617-1620
TOPO® recognition site 2: bases 1621-1625
V5 epitope: bases 1705-1746
BGH reverse priming site: bases 1784-1801
BGH polyadenylation signal: bases 1790-2014
FRT site: bases 2297-2344
Hygromycin resistance gene (no ATG): bases 2352-3372
SV40 early polyadenylation signal: bases 3504-3634
pUC origin: bases 4017-4690
bla promoter: bases 5696-5794 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4835-5695 (complementary strand)

continued on next page

Map and Features of pEF5/FRT/V5-D-TOPO®, continued

Features of pEF5/FRT/V5-D-TOPO®

pEF5/FRT/V5-D-TOPO® (5831 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human elongation factor 1α (hEF-1α) promoter	Allows overexpression of your recombinant protein in a broad range of mammalian cell types (Goldman *et al.*, 1996; Mizushima and Nagata, 1990)
T7 Promoter/priming site	Allows *in vitro* transcription in the sense orientation and sequencing through the insert
TOPO® Cloning site (directional)	Allows rapid, directional cloning of your PCR product
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of your recombinant protein with the Anti-V5 antibodies (Southern *et al.*, 1991)
BGH Reverse priming site	Allows sequencing of the insert
Bovine growth hormone (BGH) polyadenylation signal	Allows efficient transcription termination and poly-adenylation of mRNA (Goodwin and Rottman, 1992)
Flp Recombination Target (FRT) site	Encodes a 34 bp (+14 bp of non-essential) sequence that serves as the binding and cleavage site for Flp recombinase (Gronostajski and Sadowski, 1985; Jayaram, 1985; Senecoff *et al.*, 1985)
Hygromycin resistance gene (no ATG)	Allows selection of stable transfectants in mammalian cells (Gritz and Davies, 1983) when brought in frame with a promoter and an ATG initiation codon through Flp recombinase-mediated recombination via the FRT site
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

Map of pEF5/FRT/V5/GW-CAT

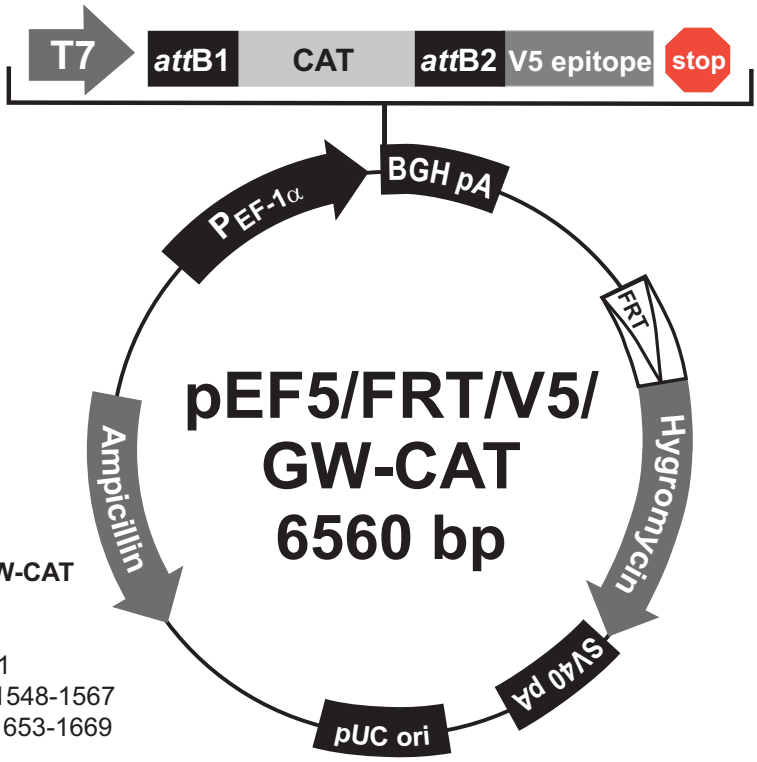
Description

pEF5/FRT/V5/GW-CAT is a 6560 bp control vector expressing chloramphenicol acetyltransferase (CAT). pEF5/FRT/V5/GW-CAT was constructed using the Gateway® LR recombination reaction between an entry clone containing the CAT gene and the pEF5/FRT/V5-DEST destination vector. For more information on Gateway® Cloning, refer to the Gateway® Technology manual which is available for downloading from our Web site (www.invitrogen.com).

CAT is expressed as a fusion to the V5 epitope tag. The molecular weight of the protein is approximately 30 kDa.

pEF5/FRT/V5/GW-CAT Map

The figure below summarizes the features of pEF5/FRT/V5/GW-CAT. **The complete sequence of pEF5/FRT/V5/GW-CAT is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 32).**



Comments for pEF5/FRT/V5/GW-CAT
6560 nucleotides

EF-1α promoter: bases 348-1531
T7 promoter/priming site: bases 1548-1567
*att*B1 recombination site: bases 1653-1669
CAT ORF: bases 1699-2355
*att*B2 recombination site: bases 2356-2381
V5 epitope: bases 2434-2475
BGH reverse priming site: bases 2513-2530
BGH polyadenylation signal: bases 2519-2743
FRT site: bases 3026-3073
Hygromycin resistance gene (no ATG): bases 3081-4101
SV40 early polyadenylation signal: bases 4233-4363
pUC origin: bases 4746-5419
bla promoter: bases 6425-6523 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 5564-6424 (complementary strand)

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:
1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add antibiotic, if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add antibiotic and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

Cell Lysis Buffer

50 mM Tris, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions. For 100 ml, combine

1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml

2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.

To prevent proteolysis, you may add 1 mM PMSF, 1 μM leupeptin, or 0.1 μM aprotinin before use.

4X SDS-PAGE Sample Buffer

1. Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol (100%)	4 ml
β-mercaptoethanol	0.8 ml
Bromophenol Blue	0.04 g
SDS	0.8 g

2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

Technical Service

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

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SDS

Safety Data Sheets (SDSs) are available at www.invitrogen.com/sds.

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Technical Service, continued

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Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 36.

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Purchaser Notification, continued

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Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Invitrogen's policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Invitrogen's commercially available Gateway® Technology.

Gateway® Entry Clones

Invitrogen understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway® Expression Clones

Invitrogen also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Invitrogen.

Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase™ from Invitrogen is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Invitrogen's Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to Invitrogen's licensing department at 760-603-7200.

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